ELISA Kit for Measuring Human ISG15

CircuLex Human ISG15 ELISA Kit

Cat# CY-8085

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Intended Use

The CycLex Research Product **CircuLex Human ISG15 ELISA Kit** is used for the quantitative measurement of human ISG15 in cell lysate, culture medium and human serum.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

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Introduction

ISG15 (Interferon (IFN)-Stimulated Gene 15) is a ubiquitin-like protein containing two ubiquitin homology domains and becomes conjugated to a variety of proteins when cells are treated with type I interferon or lipopolysaccharide. Therefore this modification (so called, ISGylation) appear to be involved in type I interferon signal transduction. Unlike ubiquitylation, ISGylation does not drive protein degradation (regulated by K48-linked ubiquitin). ISG15 is expressed as a 165 amino acid precursor that is subsequently processed to expose the C-terminal sequence LRLRGG. Like ubiquitin, this C-terminal diglycine residue is adenlylated and conjugated by a thiolester bond to sequential cystine residues on the E1-activating, E2-carrier and E3-ligase enzymes, before being transferred to lysine residues on target protein substrates.

ISG15 can also be found extracellularly in an unconjugated form (free form). Human lymphocytes and monocytes were reported to release free ISG15 following treatment with IFN-β. More than 50 % of the total ISG15 could be detected in the culture medium after 24 hours following IFN stimulation. In addition, ISG15 is highly elevated and extensively conjugated to cellular proteins in many tumors and tumor cell lines. The increased levels of ISG15 in tumor cells were found to be associated with decreased levels of polyubiquitinated proteins.

Principle of the Assay

The CycLex Research Product **CircuLex Human ISG15 ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human ISG15 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human ISG15 present. After washing away any unbound substances, an HRP conjugated antibody specific for human ISG15 is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of human ISG15. A standard curve is constructed by plotting absorbance values versus human ISG15 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

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Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 2 hours at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human ISG15 antibody
↓ Incubate for 2 hours at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-human ISG15 antibody as a capture antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing 2%Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for and sample dilution. Ready to use.

Human ISG15 Standard: One vial containing 40 ng of lyophilized recombinant human ISG15

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-human ISG15 antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

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Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation
- **Vortex mixer**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **Plate reader:** capable of measuring absorbance in 96-well plates at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **Software package facilitating data generation and analysis**: optional
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

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Precautions and Recommendations

- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residues from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain NaN_3 as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-Na_2 as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-Na_2 and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x g. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay.

Other biological samples: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

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Detailed Protocol

The CycLex Research Product **CircuLex Human ISG15 ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the human ISG15 Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer and Human ISG15 Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (ddH_2O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Human ISG15 Standard** with **1.0 mL** of **ddH_2O**. The concentration of the human ISG15 in vial should be **40 ng/mL**, which is referred as a **Master Standard** of human ISG15.

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 12 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	180 µL of Master Standard	420 µL	12.0 ng/mL
Std.2	300 µL of Std. 1 (12.0 ng/mL)	300 µL	6.0 ng/mL
Std.3	300 µL of Std. 2 (6.0 ng/mL)	300 µL	3.0ng/mL
Std.4	300 µL of Std. 3 (3.0 ng/mL)	300 µL	1.5 ng/mL
Std.5	300 µL of Std. 4 (1.5 ng/mL)	300 µL	0.75 ng/mL
Std.6	300 µL of Std. 5 (0.75 ng/mL)	300 µL	0.375 ng/mL
Std.7	300 µL of Std. 6 (0.375 ng/mL)	300 µL	0.188 ng/mL
Blank	-	300 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Master Standard should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Sample Preparation

- Optimal dilution of cell conditioned medium for measurement of ISG15 depends on cell number and IFN-does; require neat to appropriate dilution.

- Serum and plasma samples require a 10-40 fold dilution depending on ISG15 concentration in sample.

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Standard Assay Procedure for Human ISG15

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute samples with Dilution Buffer. (See "Sample Preparation" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and the **diluted samples** in duplicates, into the appropriate wells.

4. Incubate the wells **at room temperature (ca.25°C) for 2 hours**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the wells **at room temperature (ca.25°C) for 2 hours**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent** to each well. Avoid exposing the microtiter plate to direct sunlight. Covering the wells with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed.

10. Incubate the wells **at room temperature (ca.25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 3.0 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine ISG15 concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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Calculations

Average the duplicate readings for each Standard Solution, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the human ISG15 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding human ISG15 concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 0.188 ng/mL to 12 ng/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human ISG15 concentration.

Troubleshooting

1. The **Standard Solutions** should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **CircuLex Human ISG15 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted ISG15 Standard must be stored at below -70°C. The Microplate should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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Sample Preparation

Several extraction methods can be used for measurement cellular total ISG15. The following protocol has been shown to work with a number of different cell lines and is provided as an example of suitable methods. It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the cell lysate. One eight well strip of the substrate plate should be sufficient for this initial experiment. All steps of cell lysate preparation should be performed at 4°C and recovered cell lysates should be kept at below -70°C.

Preparation of Cell Lysate

A. Preparation of Cell Lysis Buffer

20 mM Tris HCl, pH 7.5, 250 mM NaCl, 10 % glycerol, 0.1 % NP-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 0.2 mM DTT

B. Preparation of poly-l-lysine coated plate

Coat the plate with 25 µg/mL poly-l-lysine (PLL) in PBS for 4-12 h at 37°C. Subsequently go to a washing step with PBS.

C. Treatment of Cells

1. Plate adherent cells in PLL-coated 96-well plate at around 1 x 10^4 cells/well.

2. Incubate the culture dish at 37°C for 7-12 hours in CO_2 incubator.

3. Add appropriate amount of test compound or IFN and vehicle for test compound to each well.

4. Incubate the culture dish at 37°C for appropriate time.

D. Cell Extraction

Note: This protocol has been successfully applied to HeLa cell line. Users should optimize the cell extraction procedure for their own applications.

1. Wash cells three times with ice-cold PBS. Remove any remaining PBS by decanting. Invert the plate and blot it against clean paper towels. At this point the cells in the plate can be frozen at below -70°C and lysed at a later date.

2. Lyse the cells by adding 0.1 mL* of Cell Lysis Buffer for 60-90 minutes at 4°C, with rotating at ca. 300 rpm by an orbital microplate shaker.

** To get a rough idea you could adjust the cell concentration to around 1-2 x 10^5 cells/mL in Cell Lysis Buffer. Resulting protein concentration of the HeLa cell lysate should be 0.03-0.04 mg/mL using this procedure.*

** The appropriate volume of Cell Lysis Buffer depends on the cell line, the cell number and the amount of total ISG15. For example, 1-2 x 10^4 HeLa cells can be lysed in 0.1 mL of Cell Lysis Buffer.*

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3. Centrifuge at 3,500 rpm for 15 minutes at 4°C using a microplate bucket. (or transfer the cell lysates to microcentrifuge tubes and centrifuge at 15,000 rpm for 5 minutes at 4°C.)

4. Transfer the clear cell lysates to new 96-well microplate or clean microcentrifuge tubes. Dilute these cell lysates 10 times with Dilution Buffer. 100 µL of these diluted cell lysates are ready for assay. Go to the section "Standard Assay Procedure for Human ISG15" at page 8.

Typical data using this protocol are shown in Fig.2 and Fig.4 (page 15 and 16)

The cell lysates can be stored at below -70°C. Avoid multiple freeze/thaw cycles. After thaw the cell lysates, Centrifuge at 15,000 rpm for 5 minutes at 4°C again since the cell lysates should be clear of any sediments or particulate matter.

NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

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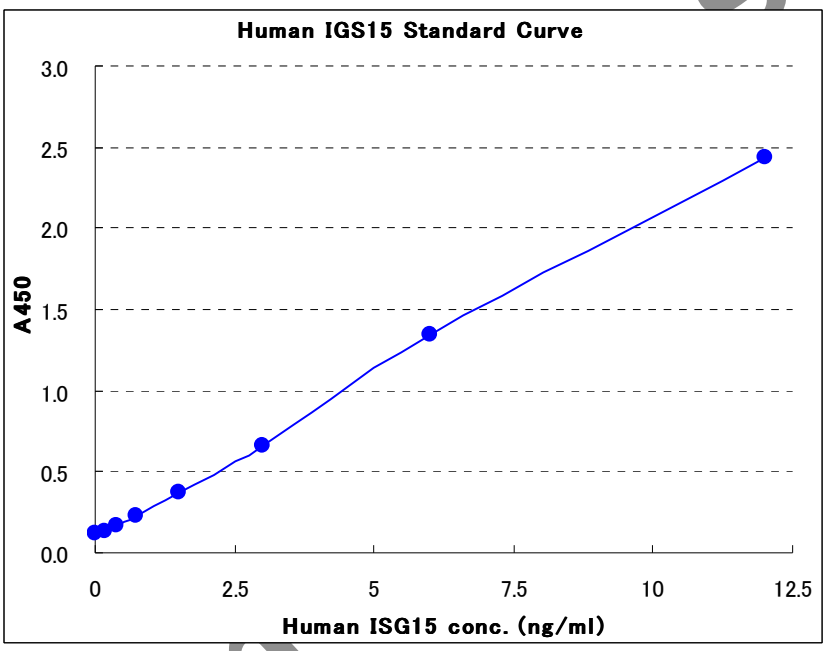
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human ISG15 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.85 ng/ml of sample.

* Dilution Buffer is pipetted into blank wells.

Typical Standard Curve



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2. Precision

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested sixteen times on one plate to assess intra-assay precision.

- Intra-assay (Within-Run, n=16) CV=1.3-4.6 %

Human ISG15 concentration (ng/mL)

	Sample 1	Sample 2	Sample 3
1	2.15	1.55	0.40
2	2.07	1.56	0.40
3	2.08	1.53	0.38
4	2.18	1.56	0.39
5	2.09	1.55	0.39
6	2.05	1.54	0.39
7	2.04	1.52	0.40
8	2.08	1.56	0.41
9	2.11	1.57	0.43
10	2.15	1.59	0.44
11	2.12	1.52	0.42
12	2.16	1.56	0.41
13	2.15	1.54	0.40
14	2.12	1.53	0.38
15	2.06	1.57	0.40
16	2.03	1.53	0.42
MAX.	**2.18**	**1.59**	**0.44**
MIN.	**2.03**	**1.52**	**0.38**
MEAN	**2.10**	**1.55**	**0.40**
S.D.	**0.05**	**0.02**	**0.02**
C.V.	**2.2%**	**1.3%**	**4.6%**

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Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in five separate assays to assess inter-assay precision.

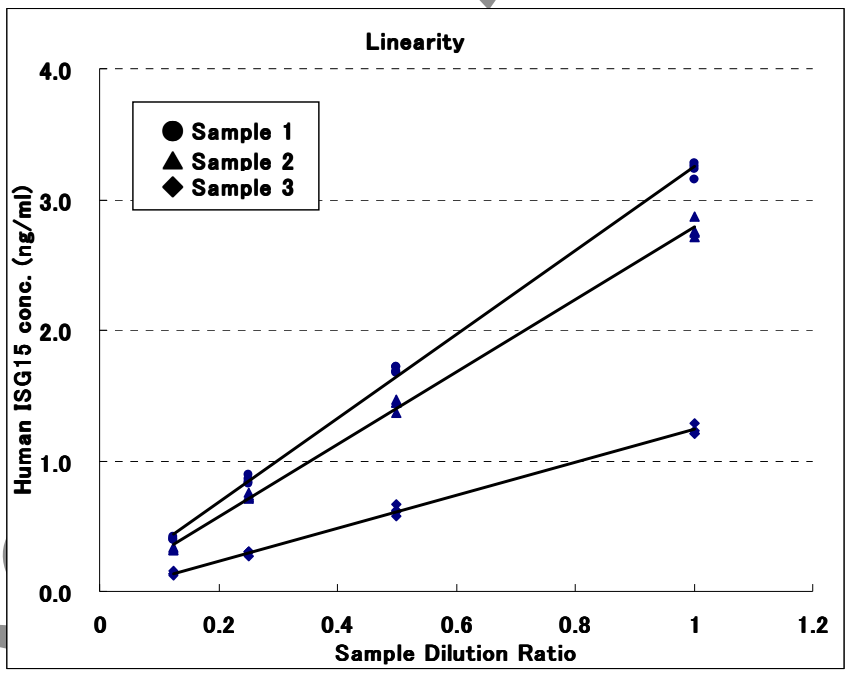
• Inter-assay (Run-to-Run, n=5), CV=2.9-7.2 %

Human ISG15 concentration (ng/mL)

	Sample 1	Sample 2	Sample 3
1	5.80	3.02	0.90
2	5.53	2.87	0.83
3	5.11	2.90	0.79
4	5.09	2.90	0.76
5	5.24	3.06	0.88
MAX.	**5.8**	**3.1**	**0.9**
MIN.	**5.1**	**2.9**	**0.8**
MEAN	**5.4**	**2.9**	**0.8**
S.D.	**0.306**	**0.084**	**0.060**
C.V.	**5.7%**	**2.9%**	**7.2%**

3. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of unconjugated (free form) human ISG15 were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.



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Example of Test Results

Fig.1 Concentrations of unconjugated (free form) human ISG15 in HeLa cell culture medium after IFNα -treatment for indicated times

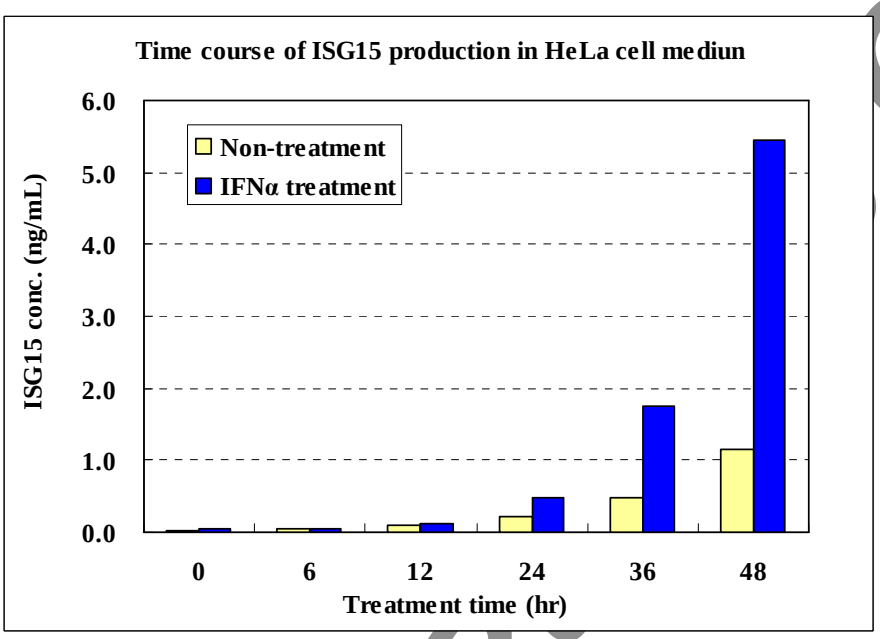

Fig.2 Concentrations of total human ISG15 in HeLa cell lysate after IFNα-treatment for indicated times

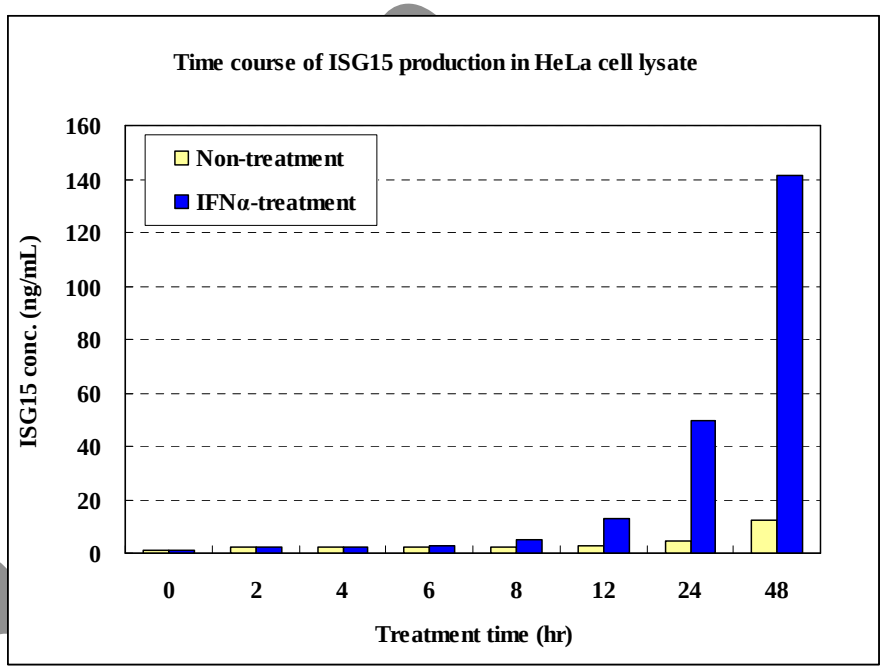

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Fig.3 IFNα-dose dependent production of ISG15 in HeLa cell culture medium

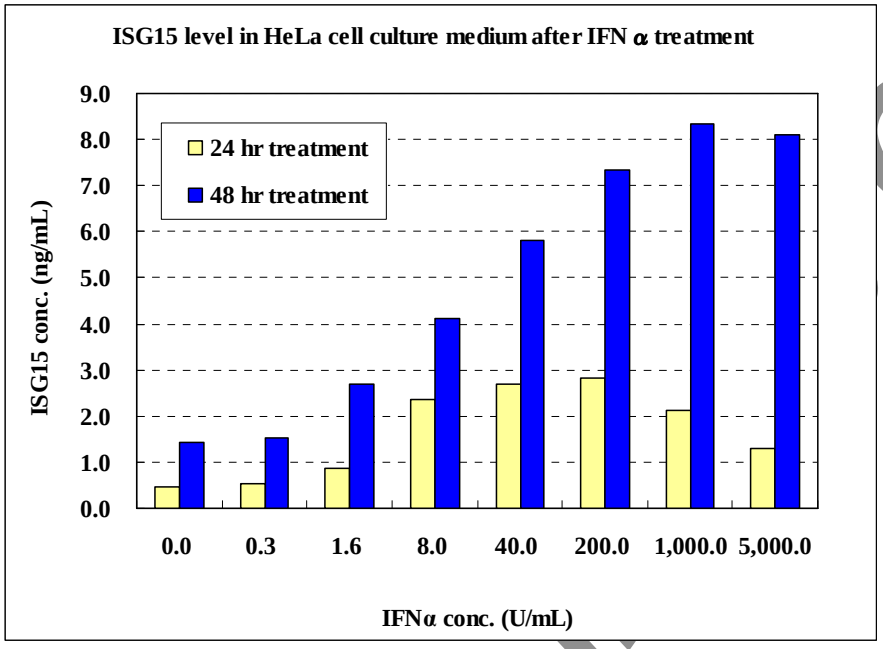
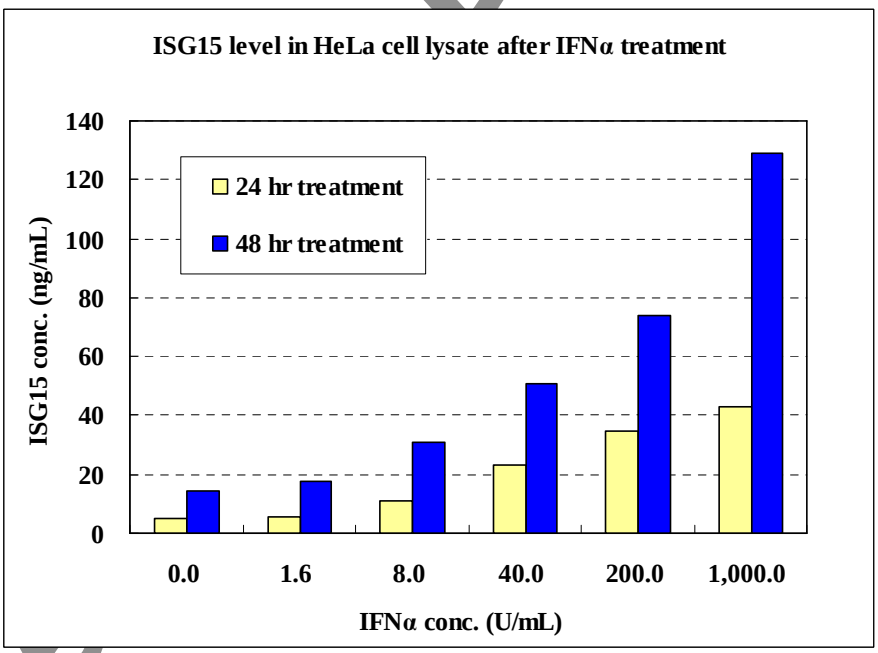


Fig.4 IFNα-dose dependent production of ISG15 in HeLa cell lysate after IFNα treatment



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Related Products

*** CircuLex Mouse ISG15 ELISA Kit: Cat# CY-8091**
*** CircuLex Human ISG15: Cat# CY-R2272**

PRODUCED BY

CycLex Co., Ltd.
1063-103 Terasawaoka
Ina, Nagano 396-0002
Japan
Fax: +81-265-76-7618
e-mail: info@cyclex.co.jp
URL: http://www.cyclex.co.jp

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